Selection in monoculture vs. mixture alters plant metabolic fingerprints

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Abstract: Aims: In grassland biodiversity experiments, positive biodiversity effects on primary productivity increase over time. Recent research has shown that differential selection in monoculture and mixed-species communities leads to the rapid emergence of monoculture and mixture types, adapted to their own biotic community. We used eight plant species selected for 8 years in such a biodiversity experiment to test if monoculture and mixture types differed in metabolic profiles using infrared spectroscopy. Methods: Fourier transform infrared spectroscopy (FTIR) was used to assess metabolic fingerprints of leaf samples of 10 individuals of each species from either monocultures or mixtures. The FTIR spectra were analyzed using multivariate procedures to assess (i) whether individuals within species could be correctly assigned to monoculture or mixture history based on the spectra alone and (ii) which parts of the spectra drive the group assignment, i.e. which metabolic groups were subject to differential selection in monocultures vs. mixtures. Important Findings: Plant individuals within each of the eight species could be classified as either from monoculture or mixture selection history based on their FTIR spectra. Different metabolic groups were differentially selected in the different species; some of them may be related to defense of pathogens accumulating more strongly in monocultures than in mixtures. The rapid selection of the monoculture and mixture types within the eight study species could have been due to a sorting-out process based on large initial genetic or epigenetic variation within the species.

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Selection in monoculture vs. mixture alters plant metabolic fingerprints

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Abstract

Aims In grassland biodiversity experiments, positive biodiversity effects on primary productivity increase over time. Recent research has shown that differential selection in monoculture and mixed-species communities leads to the rapid emergence of monoculture and mixture types, adapted to their own biotic community. We used eight plant species selected for 8 years in such a biodiversity experiment to test if monoculture and mixture types differed in metabolic profiles using infrared spectroscopy.

Methods Fourier transform infrared spectroscopy (FTIR) was used to assess metabolic fingerprints of leaf samples of 10 individuals of each species from either monocultures or mixtures. The FTIR spectra were analyzed using multivariate procedures to assess 1) whether individuals within species could be correctly assigned to monoculture or mixture history based on the spectra alone, and 2) which parts of the spectra drive the group assignment, i.e. which metabolic groups were subject to differential selection in monocultures vs. mixtures.

Important Findings Plant individuals within each of the eight species could be classified as either from monoculture or mixture selection history based on their FTIR spectra. Different metabolic groups were differentially selected in the different species; some of them may be related to defense of pathogens accumulating more strongly in monocultures than in mixtures. The rapid selection of the monoculture and mixture types within the eight study species could have been due to a sorting-out process based on large initial genetic or epigenetic variation within the species.

Introduction

Greater biodiversity in plant communities positively affects productivity and this effect can increase over time (Reich *et al.*, 2012, Tilman *et al.*, 2006). Positive biodiversity effects on productivity are often interpreted in terms of more complementary resource uptake (HilleRisLambers *et al.*, 2004, Schnitzer *et al.*, 2011, Tilman *et al.*, 2001) as plants separate along environmental niche axes (Silvertown, 2004). Additionally, plant–soil feedbacks may contribute to positive biodiversity effects on productivity by regulating plant species co-existence in plant communities (Bever, 1994, Bever, 2003, Mills *et al.*, 1998, Petermann *et al.*, 2008), leading to lower productivity at the lower end of the biodiversity gradient (Kulmatiski *et al.*, 2012, Schnitzer *et al.*, 2011). Given that there is variation in populations in ability to survive and reproduce over a range of species richness levels, it can be deduced that selection processes may occur in monocultures and mixed-species communities, which result in plant types adapted to each type of community. Recent work has demonstrated that plants can become adapted to the diversity of the community in which they grow (Lipowsky *et al.*, 2011). Furthermore, a glasshouse study has shown that selection processes can drive biodiversity effects, as reflected by cultivation of offspring derived from field monocultures (monoculture types) or mixtures (mixture types) (Zuppinger-Dingley *et al.,* 2014).

These recent findings indicate the potential for plant adaptation to the biotic environment; and, consequently, should be reflected in phenotypic differences among the selected monoculture and mixture types. We propose the novel hypothesis of selection for plant types adapted to biodiversity to explain increases in biodiversity effects over time (Zuppinger-Dingley *et al.,* 2014). In the present study we assessed metabolic fingerprints of leaf tissues with Fourier transform infrared spectroscopy (FTIR) as an indication of phenotypic differences between monoculture and mixture types in eight grassland species which had been growing for 8 years in monoculture or mixed-species communities in a large

grassland biodiversity experiment in Jena, Germany. We refer to the two types of communities as selection history.

Previously FTIR spectroscopy has been used extensively to investigate biochemical and molecular responses of algae to changes in environmental conditions (Giordano *et al.*, 2001, Giordano *et al.*, 2007) as such fingerprints give an indication of the macromolecular composition of cells (Wagner *et al.*, 2014). Although this technique has rarely been used in plant community ecology (Gidman *et al.*, 2003), FTIR fingerprinting has been used successfully to differentiate between plant genera (Gorgulu *et al.*, 2007, Kim *et al.,* 2007), to detect endophytic fungal infections in grasses (Brandl, 2011), to identify major alterations in biochemical pathways of mutant collections (Sardans *et al.*, 2011) and to determine metabolic alterations within species subjected to changes in environmental conditions (Domenighini *et al.*, 2009, Gidman *et al.*, 2003, Harmanescu *et al.*, 2012, Jones *et al.*, 2012, Lazar *et al.*, 2012, Scherling *et al.*, 2010). Plant individuals have been shown to adapt to the local biotic environment in a study focusing on plant traits across the experimental plant species richness gradient of the above-mentioned Jena Experiment (Lipowsky *et al.*, 2011). The phenotypic changes in response to the local biotic environment could have been due to genetic, epigenetic or maternal effects (Roach *et al.*, 1987, Rossiter, 1996). Fourier transform-infrared spectroscopy (FTIR) is a promising tool to determine phenotypic changes at the level of plant biochemistry. Such changes in biochemical pathways should be linked to underlying genetic or epigenetic alterations (Fiehn, 2002).

FTIR produces a biochemical signature of a selected sample (Fiehn, 2001, Johnson *et al.*, 2003) providing a snapshot of the biochemical composition of a cell (Domenighini and Giordano, 2009). This metabolic fingerprint can be used to discriminate not only between species but also between genotypes within species (Schulz *et al.*, 2007). Furthermore, because absorption peaks in FTIR spectra are due to the particular chemical bonds making up

materials (Ammann *et al.*, 2011), there is the possibility to assign peaks to specific groups of compounds such as nucleic acids, lipids or carbohydrates (Griffiths *et al.*, 1986).

We tested whether metabolic changes occured in response to selection history of either monoculture or mixture diversity over 8 years in the Jena Experiment, Germany using the distinct biochemical fingerprints produced by FTIR spectroscopy. Differences in the FTIR spectra between monoculture types or mixture types could be used as an indication of phenotypic differences via alterations in biochemical composition of the leaf samples. Furthermore, we tested whether specific wavenumber regions drove the differences between monoculture and mixture types within species.

Methods

The Jena Experiment was our source of plants from communities with a selection history of either monocultures (monoculture types) or mixtures (mixture types) in the field. This experiment was established in 2002 at a field site in Jena, Germany (50°55'N, 11°35'E, 130 m a.s.l) using 60 common Central European grassland species. Sown plant species richness ranged from 1–60 species per plot (see Roscher *et al.*, 2004 for details). We chose eight of the 60 species based on their occurrence both in plots of monocultures or mixtures for 8 years in the Jena Experiment to test if metabolic changes (changes in biochemical composition) had occurred in response to selection in monocultures vs. mixtures. Two species from each of four plant functional groups were chosen: grasses (*Festuca pratensis*, *Poa pratensis*), legumes (*Onobrychis viciifolia*, *Trifolium repens*), tall herbs (*Crepis biennis*, *Galium mollugo*) and small herbs (*Plantago lanceolata*, *Prunella vulgaris*). We collected plant cuttings from the experimental plots in Jena in April 2010 and replanted them into pots with GVZ Tref GO PP 7000 (BF4; GVZ; De Baat, Holland) substratum under glasshouse conditions to acclimatize them to the new environment before transplanting them into plots in our experimental garden at the University of Zurich with the same species combinations as

found in the original Jena plots. We propagated the plant material as cuttings of cuttings, so that the material used in the study was not directly taken from the field.

Using a JASCO 4200-FTIR instrument (Brechbühler AG, Schlieren, Switzerland) in attenuated total reflection (ATR) mode with an ATR accessory equipped with a zinc selenide (ZnSe) prism, we measured metabolic fingerprints. Whole mature leaf samples were randomly taken from plant individuals of the study species and placed onto the ATR accessory and spectra were collected (Hsu, 1997). For each leaf sample an average of 50 scans were taken with a resolution of 4 cm^{-1} using the ZnSe prism and saved for further chemometrical analyses. We used a measurement range of 650–4000 cm^{-1}. For each study species, leaf samples were taken from 10 individuals derived from 10 cuttings of different plants selected in monocultures and the same number were taken from individuals derived from plants selected in mixtures. Raw spectral data were processed with JASCO Spectra Manager 2.02.02. Each spectrum was adjusted using baseline correction (linear), ATR-correction, smoothing (Savitzky-Golay, width = 15; (Susi *et al.*, 1983), truncation (1900–650 cm^{-1}) and normalization (highest value =1, lowest value = 0). As mentioned in many biological studies (e.g., Kim *et al.*, 2007) post-measurement data treatment is needed to compare spectra and minimize inconsistences, in particular when applying second derivatives of spectra. Baseline correction eliminates baseline drifts, smoothing reduces noise, and normalization is applied to correct spectra regarding peak heights because these depend on the pressure applied by the device and might vary between different samples.

Linear discriminant analysis (LDA; Ripley, 1994) was used to calculate classification functions and assign leaf samples to their respective selection history (monocultures vs. mixtures) in a single analysis of all species (R, version 2.15.3, R Development Core Team, 2013). Canonical variate analysis (Hotelling, 1936) was used to estimate multivariate intergroup distances for each species with the Mahalanobis D-squared distance measure

(CVA; with GenStat version 16, VSN International Ldt. 2013). We used stepwise multiple regression (Hocking, 1976) to determine the selected wavenumber regions that drove the differences between monoculture and mixture types for all species in a single analysis (R, version 2.15.3, R Development Core Team, 2013). Multidimensional scaling (MDS), a multivariate method for data visualization of hidden relations among objects in data (Borg *et al.*, 2005), in the form non-metric multidimensional scaling (NMDS), was applied on the combined spectral range to determine dissimilarities among samples between each selection history ("Q-mode" analysis) for each individual species and in a single analysis of all species (R, version 2.15.3, R Development Core Team, 2013). Low stress values in NMDS analysis reflect a good fitting solution with a high degree of correspondence between the observed inter-object distances and the distances predicted by the dissimilarities. The correlation between fitted values and ordination distances was very close ($R^2 = 0.99$ for both linear and non-metric fit), with stress values ranging from 0.013 – 0.079. More detailed analyses were done in the following spectral regions broadly assigned to four groups of compounds:

Aromatic = 650–910 cm^{-1} (Hsu *et al.*, 1997),

Carbohydrate = 750–1200 cm^{-1} (Ami *et al.*, 2013)

Protein = 1500–1700 cm^{-1} (Amiali *et al.*, 2011)

Lignin = 1590–1610 cm^{-1} (Allison, 2011).

Information on peak assignments when investigating biomass derived from cyanobacteria or plants has been published earlier (see Kansiz *et al.*, 1999, Gorgulu *et al.*, 2007). We focused on these spectral regions to determine if specific wavenumber regions could be associated with monoculture or mixture selection history. Applying the two orthogonal ordination axes from the NMDS analysis of all species with selection history as binary response variable in generalized mixed models (Breslow *et al.*, 1993, Wolfinger *et al.*, 1993); GenStat ,version 16, VSN International Ldt. 2013), we tested if plants selected in either monoculture or mixture

communities over 8 years showed distinct metabolic fingerprints. The results, calculated for the full range of wavenumbers ("Fingerprint") and for the four specific regions listed above were summarized in analyses of variance (ANOVA) tables. Significance tests were based on approximate F-tests using appropriate error terms and denominator degrees of freedom. The fixed terms in the models were: selection history (monocultures vs. mixtures), species and the interaction between these. Species and plant sample were used as random terms.

The second derivative of the corrected spectra, allowing for band narrowing and therefore distinguishing more features, was then calculated (Savitzky-Golay, width = 15; Susi and Michael Byler, 1983). Hierarchical cluster analysis, using the complete linkage method with Euclidean distance (Everitt, 1974, Hartigan, 1975); R, version 2.15.3, R Development Core Team, 2013), was used to determine which samples were most alike and therefore would cluster together and how well these clusters represented the selection history of the species.

Results

Selection history clearly altered the metabolic fingerprints of the species in our study. The matrix produced using LDA showed that plant individuals were 99% correctly classified as belonging to either monoculture or mixture selection history (Table 1). Two of the species, *Plantago lanceolata* and *Poa pratensis*, accounted for the 1% failures in the assignment of individuals to monoculture or mixture selection history. Using specific wavenumber regions related to proteins, carbohydrates or aromatics (Fig. 1) we obtained similar levels of accuracy, i.e. 99% correct assignment to monoculture or mixture selection history. Only the wavenumber region associated with lignin (Fig. 1; Table S1) assigned a lower number of individuals correctly to the respective selection history. Certain specific wavenumbers with significantly different absorption between monoculture and mixture plant types could tentatively be associated with specific biochemical compounds (Table 2).

Additional evidence for a shift in metabolic fingerprints with selection history was provided by the separate analyses for each species using Mahalanobis distances for the four wavenumber groups mentioned above, showing that the maximum distance was always between plant individuals from different selection histories (Table 3; Fig. S1). The greatest distance between the two selection histories was found across all wavenumbers combined or in the protein wavenumber region in *Prunella vulgaris*; in the aromatic and lignin wavenumber regions *Trifolium repens* and *Onobrychis viciifolia*, respectively, showed the greatest distance between monoculture and mixture types. In the carbohydrate wavenumber region, *Galium mollugo* showed the greatest distance between the two selection histories.

NMDS-ordinations based on Euclidean distance dissimilarities calculated between the 20 individuals of each of the eight species showed that individuals with monoculture selection history were clearly separated from individuals with mixture selection history in the ordination plots for each of the eight species (Fig. 2). Stress values of under 0.075 indicate a high degree of correspondence between the observed inter-object distances and the distances predicted by the dissimilarities. Mixed effects models using the combined data set of all eight species (Table 4) showed that all species differed significantly in their FTIR spectra ($P = <0.001$). Differences between monoculture and mixture selection history were in part common to all species (significant main effects of selection history, Table 4) but additionally highly species-specific (significant interactions, Table 4). These results show that eight years of selection in monocultures vs. mixtures has led to clearly differentiated metabolic fingerprints in the eight studied grassland species.

Finally, cluster analysis on the second derivative of spectra for each species again clearly differentiated between monoculture types and mixture types for most species, with individuals of common selection history generally clustering together (Fig. 3; Fig. S2). Monoculture types clustered strongly into single groups for the two tall herbs *Galium*

mollugo and *Crepis biennis*, as well as for the two legumes *Onobrychis viciifolia* and *Trifolium repens*. In contrast, mixture types clustered strongly for the two grasses *Festuca pratensis* and *Poa pratensis*. The small herb *Prunella vulgaris* showed the weakest separation between monoculture and mixture types whereas the other small herb *Plantago lanceolata* showed stronger clustering for monoculture history.

Discussion

We determined metabolic alterations occurring after eight years of selection in plant communities of monocultures or mixed-species diversity in the Jena Experiment, Germany. These metabolic alterations show that plants with different biochemical features have been selected in monoculture vs. mixed-species communities. Currently, we cannot say whether the response to selection was based on different plant genotypes occurring in the populations of the study species or if the phenotypic differences reflect differential epigenetic or maternal carryover effects. Independent of the mechanisms, it also appeared that selection was stronger in monocultures than in mixtures because clustering of spectra was tighter among plant individuals with monoculture than with mixture selection history. This may have been related to larger population sizes in experimental plots harboring only one rather than several species or to stronger selection pressures exerted e.g. by pathogen accumulation in monocultures (Magarey, 1999, Petermann *et al.*, 2008).

Similar alterations in metabolic responses of vascular plants to environmental conditions have been reported in other studies. FTIR spectroscopy identified metabolic differences in tomato fruits from plants that were grown either under normal conditions or subject to salinity stress (Johnson *et al.*, 2003, Smith *et al*., 2003). Additionally, tomato plants showed metabolic alterations of leaf tissue in response to nitrogen nutrition under two different light intensities (Urbanczyk-Wochniak *et al*., 2005). In grassland plants, alterations in metabolic fingerprints have been observed in response to different types of fertilizer

(Harmanescu *et al.*, 2012). Metabolic fingerprints can also reflect biotic interactions, as the effects of grazing on the grass *Deschampsia flexuosa* could be detected using FTIR (Jones *et al.*, 2012). The above studies mainly reflect plastic responses of genotypes to growth in different environments, whereas in our study the metabolic changes reflect differences among plants with different selection history but grown in a common environment. This indicates that the described changes are heritable, although this heritability may be due to genetic or epigenetic differences or to persistent maternal carry-over effects. Recently, evidence for variation in metabolic patterns associated with species richness was found for three plant species, *Lotus corniculatus*, *Bellis perennis* and *Leontodon autumnalis*, after six years in the Jena Experiment in plots of 1, 2, 4, 8, 16 and 60 species (Scherling *et al.*, 2010). Although in this case the plants were observed *in situ* in the different biotic environments and differences could therefore have been purely plastic, these results are consistent with our findings. Thus, for those three species it is conceivable that the responses reflected more than plastic adjustments of individual genotypes, namely selection of different genotypes in the different environments.

Genetically-caused differences in FTIR fingerprints have been reported between *Arabidopsis thaliana* mutants and wild-types (Fiehn, 2002) and in the same species metabolomics have been used to differentiate genotypes (Macel *et al.*, 2010). In a study with *Acantholimon*, *Astragalus*, and *Ranunculus* species, not only were clusters representing the three genera produced using FTIR fingerprints of their leaves, but also such fingerprints differentiated subgroups of species according to the source geographical regions (Gorgulu *et al.*, 2007). Similarly, in a field study using the Douglas fir, *Pseudotsuga menziesii*, a strong signal environmental variation could be shown in metabolite profiling, despite a weak signal of genetic variation (Robinson *et al.*, 2007).

Although we tentatively assigned compounds to the wavenumbers significantly contributing to variation between the two selection histories of monocultures vs. mixtures, further studies are needed to identify specific compounds underlying the possible adaptations to the specific biotic environments. Furthermore, despite hypothesizing that the differences we observed may be due to epigenetic or maternal effects, we suggest that they are more likely based on differential selection of genotypes. As our study species were all perennials with rare establishment of new plants from seeds, thus the result implies that the original plant material used to establish the Jena Experiment contained a large amount of standing genetic (or epigenetic) variation from which pre-adapted genotypes (or epigenetic variants) could be selected by a sorting process. Together with the occasional recombination event during sexual reproduction and subsequent seedling recruitment this would then have allowed for the rapid evolution of monoculture and mixture types.

An important selection factor in monocultures could have been negative plant–soil feedbacks. Soil organisms affect plant performance (Bever *et al*. 1997) and plant associations with the soil microbial community via plant–soil feedback mechanisms can alter soil community composition (van der Putten 1997, Kardol *et al*. 2007). Because such plant–soil and plant–plant interactions are considered key for the maintenance of species diversity in grassland ecosystems (Petermann *et al*. 2008) we suggest that the observed differences in biochemical composition between monoculture and mixture types may have resulted from co-evolution of the plants with soil biota.

We demonstrated that alterations in biochemical composition can change the metabolic fingerprint of eight species selected in monocultures or in mixtures over 8 years. Plant individuals within each of the eight species could be classified as either from monoculture or mixture selection history based on their FTIR spectra, indicating that within such communities there may be selection for different biochemical features.

Supplementary Data

We provide additional information on classification of individuals into mixture and monoculture types based on each of the main biochemical classes, aromatics, carbohydrates, protein, and lignin, in Table S1. Figure S1 shows FTIR spectra for each of the eight species, while Figure S2 shows cluster dendrograms for the six species not shown in the main text (see Figure 3).

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Table 1: LDA matrix of a single analysis of eight grassland species, of the predicted classification into monoculture or mixture selection history using absorbance values in the range of wavenumbers from 650–1900 cm^{-1} derived from FTIR spectroscopy.

Species		*Crepis biennis*		*Festuca pratensis*		*Galium mollugo*		*Onobrychis viciifolia*		*Plantago lanceolata*		*Poa pratensis*		*Prunella vulgaris*		*Trifolium repens*	
	History	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture
Crepis biennis	Mixture	**9**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	**1**	**9**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Festuca pratensis	Mixture	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0	0	0
Galium mollugo	Mixture	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0
Onobrychis viciifolia	Mixture	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0
Plantago lanceolata	Mixture	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0
Poa pratensis	Mixture	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	**1**	**9**	0	0	0	0
Prunella vulgaris	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0
Trifolium repens	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**

Table 2: The most significant wavenumbers ($P < 0.001$ without correction for multiple testing) in the range 650–1900 cm^{-1} differentiating plant individuals from monoculture vs. mixture selection history in a single analysis of eight grassland species using FTIR, with biochemical compounds tentatively assigned to these wavenumbers (Baseri *et al.*, 2011, Coates, 2000, Movasaghi *et al.*, 2008, Stuart, 1996).

Wave-number (cm^{-1})	Difference in absorbance values	Standard error of difference	*t*-value	Compound
756	-4.31	1.24	-3.47	Aliphatic chloro
804	3.40	0.95	3.60	Left-handed helix DNA
814	-3.70	1.06	-3.48	Epoxy and oxirane rings
872	2.77	0.76	3.65	Epoxy and oxirane rings
881	-4.91	1.07	-4.60	Epoxy and oxirane rings
1016	-5.22	1.39	-3.76	Glycogen
1026	7.58	2.21	3.43	Glycogen
1036	-6.04	1.38	-4.39	Glycogen
1065	5.29	1.40	3.79	Protein amide I
1084	8.44	2.30	3.67	Protein amide I
1257	-11.68	3.18	-3.68	Phospholipids
1267	13.90	3.49	3.98	Phospholipids
1277	-12.00	3.16	-3.80	Phospholipids
1315	-4.99	1.23	-4.05	Aromatic amine
1325	12.21	2.04	5.99	Aromatic amine
1335	-12.82	2.51	-5.11	Polysaccharides, pectin
1431	-9.85	2.50	-3.93	Methylene, methyl groups
1450	10.03	1.71	5.87	Methylene
1460	-4.99	1.15	-4.35	Benzene ring
1489	-10.06	2.32	-4.33	Amide II
1566	17.61	3.80	4.63	Aromatic ring
1576	-13.95	3.53	-3.95	Adenine
1662	16.89	4.50	3.75	Alkenyl (lipids)
1720	-5.15	1.25	-4.10	Ester group
1730	12.25	2.01	6.10	Fatty acid ester
1740	-18.05	3.36	-5.37	Aliphatic ester
1749	15.63	3.18	4.92	Aliphatic ester
1759	-7.68	1.90	-4.03	Alkyl carbonate
1884	-4.54	0.61	-7.50	Carbonyl
1894	4.09	0.57	7.12	Carbonyl

Table 3: Maximum Mahalanobis (D-squared) distance between individuals selected in monocultures vs. mixtures in a biodiversity experiment in Jena, Germany. The eight species were analyzed separately using CVA with absorbance values from FTIR spectra for wavenumbers in the regions assigned to aromatics (650–910 cm^{-1}), carbohydrates (750–1200 cm^{-1}), proteins (1500–1700 cm^{-1}) and lignins (1590–1610 cm^{-1}).

Species	*Crepis biennis*	*Festuca pratensis*	*Galium mollugo*	*Onobrychis viciifolia*	*Plantago lanceolata*	*Poa pratensis*	*Prunella vulgaris*	*Trifolium repens*
FTIR wavenumber (cm^{-1})								
650 - 1900	17.88	7.81	33.19	17.92	20.34	7.60	26.58	17.88
650 - 910	4.98	8.42	9.08	13.24	4.88	3.94	8.88	23.42
750 - 1200	10.01	7.40	33.19	17.92	4.88	3.29	18.36	4.68
1500 - 1700	5.41	9.88	8.31	14.63	14.63	7.60	23.38	1.82
1590 - 1610	2.59	5.29	6.10	17.98	2.63	5.61	5.79	3.71

Table 4: Results of mixed-effects ANOVA for plants selected in monocultures vs. mixtures over 8 years in a biodiversity experiment in Jena, Germany. MDS axes ("MDS1", "MDS2") calculated from FTIR absorbance values using NMDS analysis were used as dependent variables. Abbreviations: numDf = degrees of freedom of term, denDf = degrees of freedom of error term, F statistic = variance ratio, *P* = significance level).

MDS1			Fingerprint		Aromatics		Carbohydrates		Proteins		Lignin	
Fixed term	numDf	denDf	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Species (Sp)	7	72	94.36	**<0.001**	118.27	**<0.001**	56.04	**<0.001**	174.70	**<0.001**	56.04	**<0.001**
Selection history (SH)	1	72	10.97	**0.001**	0.02	0.879	11.73	**0.001**	0.69	0.407	11.73	**0.001**
Sp × SH	7	72	9.83	**<0.001**	2.89	**0.010**	8.91	**<0.001**	11.25	**<0.001**	8.91	**<0.001**
MDS2												
Fixed term	numDf	denDf	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Species (Sp)	7	72	90.50	**<0.001**	35.31	**<0.001**	185.72	**<0.001**	142.56	**<0.001**	185.72	**<0.001**
Selection history (SH)	1	72	3.53	0.064	1.13	0.291	3.33	0.072	2.50	0.118	3.33	0.072
Sp × SH	7	72	6.27	**<0.001**	8.66	**<0.001**	0.83	0.564	25.54	**<0.001**	0.83	0.564

Figure 1: Mean FTIR spectra wavenumber of eight European grassland species selected in monocultures or mixtures (n = 80 each) over 8 years in a biodiversity experiment in Jena, Germany, showing the variation in the metabolic fingerprint between monoculture and mixture selection history.

Figure 2: NMDS ordination plot based on Euclidean distance dissimilarities of FTIR spectra of leaves from individuals for each of eight central European grassland species selected in monocultures or mixtures over 8 years in a biodiversity experiment in Jena, Germany.

Figure 3: Dendrogram of individuals of two of eight grassland species selected in monocultures or mixtures over 8 years in a biodiversity experiment in Jena, Germany, based on values of the second derivative of FTIR spectra (dendrograms for the other six species are presented in Fig. 2 of the Appendix).

Figure 1

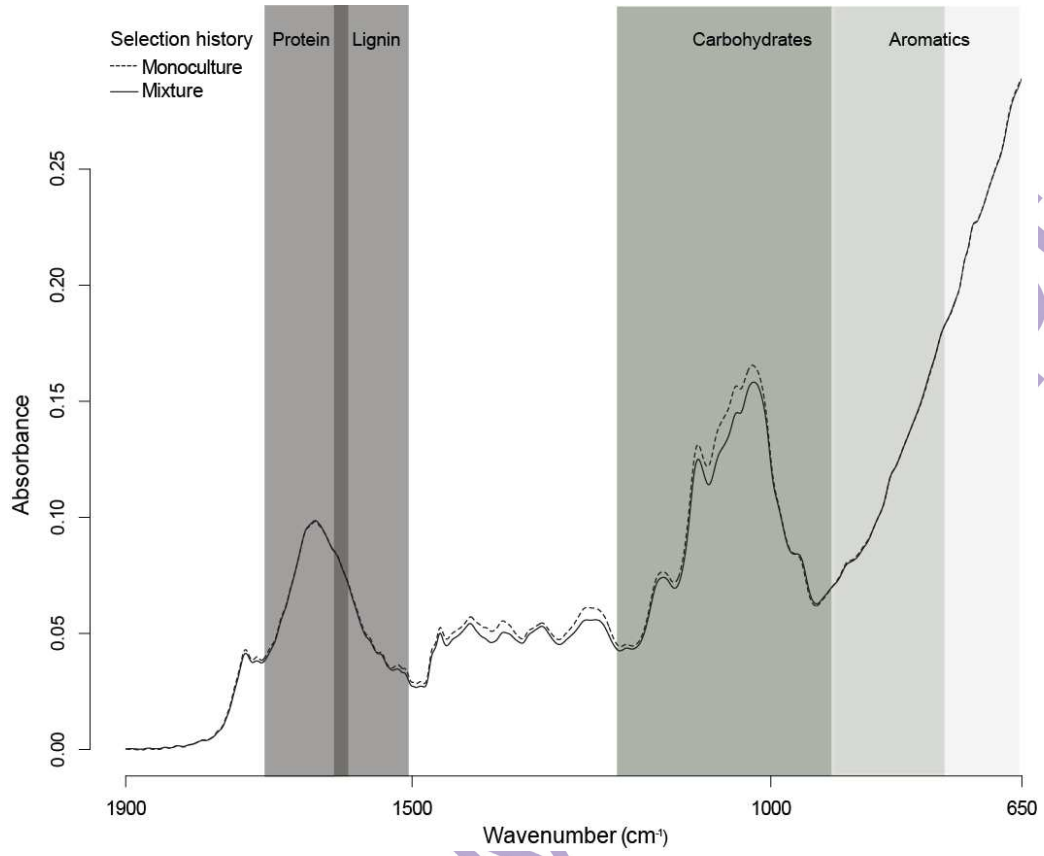


Figure 2

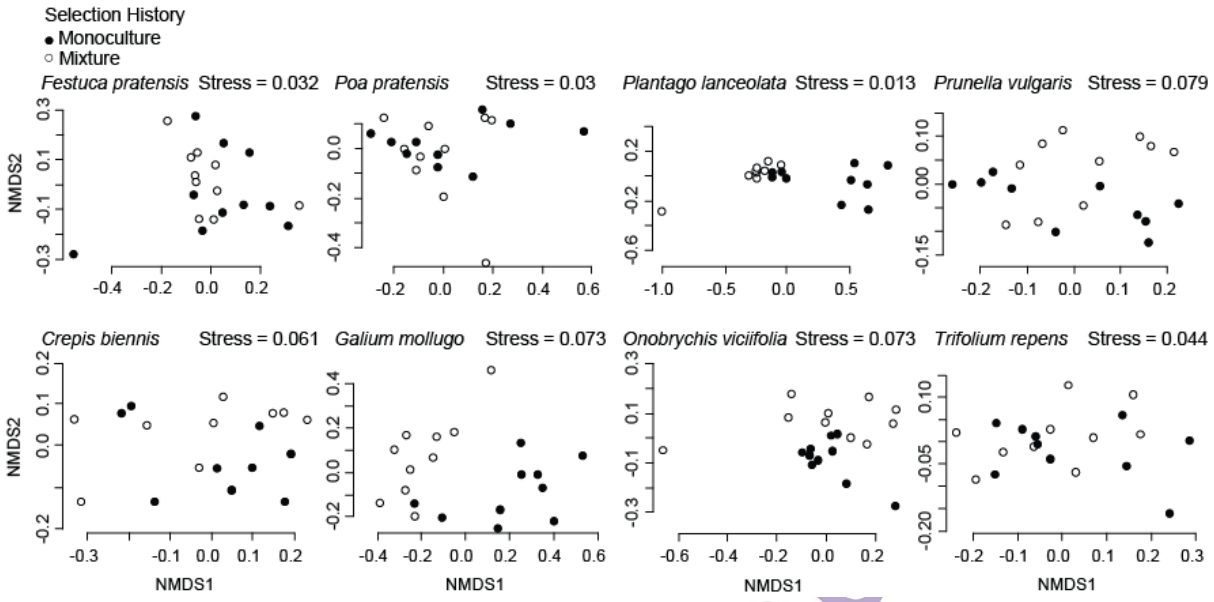
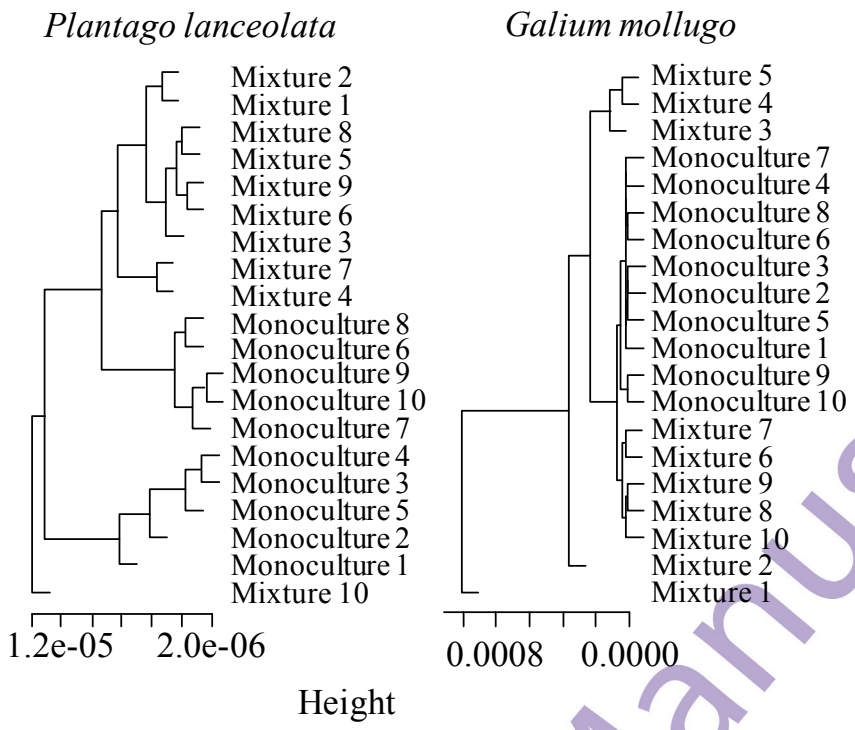


Figure 3



Supplementary Data

Table S1: LDA matrix of a single analysis of eight grassland species with the predicted classification into monoculture or mixture selection history for the FTIR wavenumbers for aromatics (650–910 cm^{-1}), carbohydrates (750–1200 cm^{-1}), proteins (1500–1700 cm^{-1}) and lignins (1590–1610 cm^{-1}); spectral data were collected using FTIR.

Species		*Crepis biennis*		*Festuca pratensis*		*Galium mollugo*		*Onobrychis viciifolia*		*Plantago lanceolata*		*Poa pratensis*		*Prunella vulgaris*		*Trifolium repens*	
	History	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture
Crepis biennis	Mixture	**9**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	**0**	**10**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Festuca pratensis	Mixture	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0	0	0
Galium mollugo	Mixture	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0
Onobrychis viciifolia	Mixture	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0
Plantago lanceolata	Mixture	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0
Poa pratensis	Mixture	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0
Prunella vulgaris	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0
Trifolium repens	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**
Crepis biennis	Mixture	**10**	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	**1**	**9**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Festuca pratensis	Mixture	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0	0	0
Galium mollugo	Mixture	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0
Onobrychis viciifolia	Mixture	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0
Plantago lanceolata	Mixture	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0
Poa pratensis	Mixture	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0
Prunella vulgaris	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0
Trifolium repens	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**

Species	History	*Crepis biennis*		*Festuca pratensis*		*Galium mollugo*		*Onobrychis viciifolia*		*Plantago lanceolata*		*Poa pratensis*		*Prunella vulgaris*		*Trifolium repens*	
		Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture	Monoculture
Crepis biennis	Mixture	**9**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	**0**	**10**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Festuca pratensis	Mixture	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0	0	0
Galium mollugo	Mixture	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0	0	0
Onobrychis viciifolia	Mixture	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0
Plantago lanceolata	Mixture	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0
Poa pratensis	Mixture	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0
Prunella vulgaris	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**	0	0
Trifolium repens	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**10**	**0**
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**
Crepis biennis	Mixture	**9**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	**1**	**8**	0	0	0	0	0	0	0	0	0	0	0	0	0	*1*
Festuca pratensis	Mixture	0	0	**8**	**2**	0	0	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	**2**	**8**	0	0	0	0	0	0	0	0	0	0	0	0
Galium mollugo	Mixture	0	0	0	0	**10**	**0**	0	0	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	**2**	**7**	0	0	0	0	0	0	*1*	0	0	0
Onobrychis viciifolia	Mixture	0	0	*1*	0	0	0	**7**	**2**	0	0	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0	0	0
Plantago lanceolata	Mixture	0	0	0	0	0	0	*1*	0	**9**	**0**	0	0	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	**0**	**10**	0	0	0	0	0	0
Poa pratensis	Mixture	0	0	0	0	0	0	0	0	0	0	**8**	**2**	0	0	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	**1**	**9**	0	0	0	0
Prunella vulgaris	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	**9**	**1**	0	0
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	**2**	**8**	0	0
Trifolium repens	Mixture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**6**	**4**
	Monoculture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**10**

Figure S1: Mean FTIR spectra (n = 10 plant individuals for each curve) for eight European grassland species selected in monocultures or mixtures over 8 years in a biodiversity experiment in Jena, Germany.

Figure S2: Dendrogram of individuals of six species selected in monocultures or mixtures over 8 years in a biodiversity experiment in Jena, Germany, based on values of the second derivative of FTIR spectra (dendrograms for the other two species are presented in Fig. 3 of the main text).

Figure S1

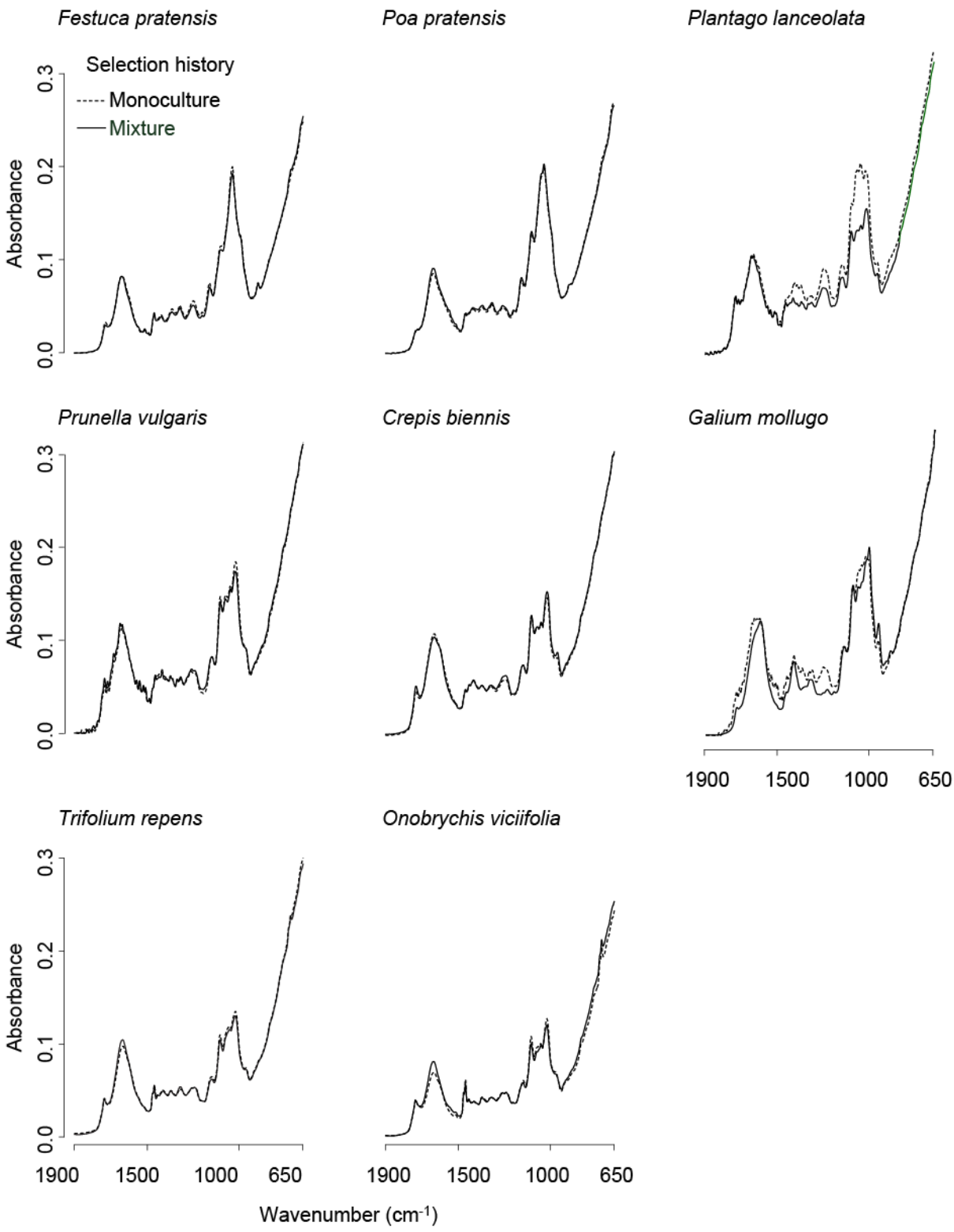

Festuca pratensis
Poa pratensis
Plantago lanceolata
Selection history
Monoculture
Mixture
Absorbance
0.0
0.1
0.2
0.3
Prunella vulgaris
Crepis biennis
Galium mollugo
Trifolium repens
Onobrychis viciifolia
1900
1500
1000
650
Wavenumber (cm-1)

Figure S2